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General and Comparative Endocrinology 133 (2003) 61–70

GENERAL AND COMPARATIVE ENDOCRINOLOGY

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Dexamethasone induced preadipocyte recruitment and expression of CCAAT/enhancing binding protein α and peroxisome proliferator activated receptor-γ proteins in porcine stromal-vascular (S-V) cell cultures obtained before and after the onset of fetal adipogenesis☆

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Accepted 4 April 2003

Abstract

The present study examined the influence of dexamethasone (DEX) treatment on preadipocyte recruitment and expression of transcription factor proteins in adipose tissue stromal-vascular (S-V) cell cultures from 50 and 75 day old pig fetuses and young pigs. C/EBPα, C/EBPδ, and PPARγ immunoreactive cells had evenly reactive nuclei and unreactive nucleoli. DEX recruited many more preadipocytes in 75 day than in 50 day fetal S-V cultures. However, DEX did not increase the number of differentiated preadipocytes (lipid+, C/EBPα+) in 50 day S-V cultures and only slightly increased this number in 75 day fetal S-V cultures. In fetal cultures, extensive, precocious increases in C/EBPα expression (number of reactive cells) by day three were followed by extensive decreases in expression. However, PPARγ expression was not expressed precociously since preadipocyte lipid accretion and PPARγ immunoreactivity were strongly linked in fetal and pig S-V cultures. Nevertheless, all cells with lipid in fetal S-V cultures were C/EBPα and PPARγ reactive. DEX increases preadipocyte differentiation in pig S-V cultures and in this study DEX increased PPARγ expression to a much greater degree in pig than in fetal S-V cultures. These studies suggest that restricted adipogenesis in the pig fetus is attributable to limited DEX induced PPARγ expression.
Published by Elsevier Science (USA).

Keywords: Preadipocyte; Fetus; Transcription factors; Immunocytochemistry; Pig; Differentiation

1. Introduction

Comprehensive studies of 3T3 preadipocyte cell lines have examined the roles of a number of transcription factors during differentiation including PPARγ and C/EBPα (reviews, Fajas et al., 1998, 2001). Interdependence or cross-regulation between PPARγ and C/EBPα is required for preadipocyte differentiation and maintenance of the fully differentiated adipocyte phenotype (reviews, Fajas et al., 1998, 2001). The relationship between these transcription factors during porcine adipogenesis has not been resolved since pig S-V cell cultures express significant quantities of C/EBPα and PPARγ protein and mRNA on the first day of culture, well before dexamethasone (DEX) or glucocorticoid (Ding et al., 1999; Hausman, 2001; Yu and Hausman, 1998) induced adipogenesis. Furthermore, C/EBPα and PPARγ proteins were detected in fetal pig adipose tissue before the onset of adipogenesis (Kim et al., 2000; Lee et al., 1998). Therefore, the developmental and temporal relationship between PPARγ and C/EBPα is obscured by high levels of expression before the onset of adipogenesis in the fetus and in pig S-V cultures.

In contrast to pig S-V cultures, S-V cell cultures from 105 day fetuses expressed low levels of C/EBPα protein

☆ Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the USDA and does not imply its approval to the exclusion of other products that may be suitable. The authors gratefully acknowledge the excellent technical execution of these studies by Rosetta Gaines and Sylvia Po.

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0016-6480/$ - see front matter. Published by Elsevier Science (USA).
doi:10.1016/S0016-6480(03)00149-7

on the first day of culture (Hausman and Yu, 1998; Yu and Hausman, 1998). A DEX + insulin + FBS protocol induced the histogenesis of a number of fat cells in S-V cultures from 110 and 75 day fetuses but induced very few fat cells in S-V cultures from 50 day fetuses (Hausman, 1992). Apparently, the number of preadipocytes recruited by glucocorticoids is very low at 50 days but increases to maximum levels by 75 day of development. However, preadipocyte recruitment in cultures from 75 day fetuses is associated with much lower lipogenesis than in cultures from 110 day fetuses and young pigs (Hausman, 1992). Examination of distinct stages of preadipocyte differentiation should facilitate the identification of temporal patterns of C/EBPα and PPARγ protein expression during porcine adipocyte differentiation. Therefore, in the present study we examined C/EBPα and PPARγ protein expression during a period of little to no preadipocyte recruitment and lipogenesis (50 day fetal S-V cultures), a period of significant preadipocyte recruitment but little to no lipogenesis (75 day fetal S-V cultures) and during a period of significant preadipocyte recruitment and lipogenesis (young pig S-V cell cultures). These studies should identify the patterns of expression of C/EBPα and PPARγ proteins relative to preadipocyte lipid accretion during porcine adipocyte differentiation. The developmental relationship between the initial expression of C/EBPα and PPARγ proteins may regulate preadipocyte lipid accretion and differentiation in porcine S-V cell cultures.

2. Materials and methods

2.1. Cultures of S-V cells from fetal and postnatal pigs

In the present study we used early and late DEX treatment protocols to increase the proportion of preadipocytes in fetal S-V cultures (Hausman and Richardson, 1998; Yu and Hausman, 1998). Subcutaneous adipose tissue was removed aseptically and digested with collagenase as described elsewhere (Hausman et al., 1984; Hausman, 1989). Aliquots of S-V cells were stained with Rappaport's stain and counted on a hemocytometer. One pool of fetal S-V cells was obtained from each dam with 2–8 fetuses providing cells. We collected and studied four pools of S-V cells from 50 day fetuses and nine pools of S-V cells from 75 day fetuses.We also studied four cultures of adipose tissue S-V cells derived from each of four young pigs (5–7 days old). Additionally, cultures of S-V cells from semitendinosus muscles were established from each of three young pigs. Both muscles from each pig were excised and all visible connective tissue was removed from the excised muscles prior to mincing and processing with a conventional collagenase digestion protocol used to establish adipose tissue S-V cell cultures (Hausman et al., 2002). Cells were seeded in 35 mm tissue culture dishes containing 2 ml of seeding medium at a density of 10^4/cm^2. Seeding medium consisted of Dulbecco's modified Eagle's nutrient mixture F-12: Ham's (Sigma, St. Louis, MI), 5 mM glucose, 40 mg/L gentamicin sulfate, 50 mg/L cephalothin, 2.5 mg/L Amphotericin B (Gibco), and 10% fetal bovine serum (FBS) with or without 80 nM DEX. Compared to FBS alone, treatment with FBS + DEX (day 0–3) enriched for preadipocytes by 10- to 20-fold in fetal and young pig S-V cell cultures (Yu and Hausman, 1998; Yu et al., 1997). The high level of DEX, 80 nM, in in this protocol optimizes preadipocyte enrichment (Yu et al., 1997). Cultures were switched to a serum-free medium which included a mixture of insulin, transferrin, and selenium (ITS, Sigma Chemical) for day 3–6. We utilized three protocols: (1) FBS + DEX, day 0–3 followed by ITS day 3–6; day 3 and day 6 of this "early" DEX treatment protocol are identified as ED-3 and ED-6, respectively, (2) FBS, day 0–3 followed by ITS + 10 nM DEX day 3–6; day 6 of this "late" DEX treatment protocol is identified as LD-6; and (3) FBS, day 0–3 followed by ITS day 3–6; days 1, 3, and 6 of this control or no DEX treatment protocol are identified as C-1, C-3, and C-6, respectively. Media were changed every 3 days.

2.2. Histochemistry and evaluation of immunoreactive cells, fat cells, and total cell number

Cultures were routinely stained for lipid and counterstained as detailed elsewhere (Hausman, 1981). Three photomicrographs of each vessel were used for total cell counting. Fat cells were counted in photomicrographs of large fields (2.2 mm^2) whereas immunoreactive cells were counted in photographs of smaller fields, either .33 or 1.3 mm^2 depending on the time in culture and antigen. Regardless, 10–15 photographs/microscopic fields of each dish were counted.

2.3. Immunochemistry

We stained for the AD-3 monoclonal antibody to identify or mark preadipocytes as described (Hausman and Richardson, 1998). The AD-3 antibody is an anti-pig adipocyte antibody that recognizes a surface antigen on adipocytes and preadipocytes (Wright and Hausman, 1990a, 1990b). The AD-3 antigen is expressed by preadipocytes before overt differentiation (Wright and Hausman, 1990a, 1990b). Cultures were fixed and reacted with AD-3 (1/50 of hybridoma supernatant) and stained with FITC anti-mouse IgG or an ExtrAvidin Peroxidase staining kit (Sigma Chemical). Mouse PPARγ 1,2 antibody and C/EBPα, and C/EBPδ antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The epitope for the C/EBPδ

antibody, Catalogue # sc-151, corresponds to an 18–20 amino acid sequence mapping at the carboxy terminus of rat C/EBPδ. The epitope for the C/EBPα antibody, Catalogue # sc-61, corresponds to amino acids 253–265 of rat C/EBPα. These antibodies are reactive for the mouse, rat, and human. The epitope for the monoclonal PPARγ 1,2 antibody, Catalogue # sc-7273, maps at the carboxy terminus of human PPARγ. Rabbit polyclonal antibody raised against mouse PPARγ 1,2 was purchased from Affinity BioReagents (Golden, CO; Catalogue # PA3-821). Immunocytochemistry was performed as described (Yu and Hausman, 1998). Briefly, cultures were washed three times with 0.01 M of PBS and fixed with 4% paraformaldehyde for 30 min. The cultures for C/EBP and PPARγ staining were permeabilized with PBS containing 3% of Triton X-100 for 15 min and then incubated with either anti-C/EBP antibodies (1/50–1/500) or anti-PPARγ antibody (1/50–1/250). Reactivity was visualized by using either a FITC conjugated second antibody (1/200; Sigma Chemical, St. Louis) or an Extravidin Peroxidase Staining Kit (Sigma Chemical, St, Louis, MO). The staining kits were used as recommended by suppliers. Staining for the C/EBPα and δ Santa Cruz antibodies was blocked by a 2 h pre-incubation with control peptides (Santa Cruz Biotechnology) for the respective antibodies. Use of an unrelated primary antibody or second antibody alone showed either no fluorescence or little to no peroxidase staining. In earlier studies of young pig S-V cultures, immunoreactivity for the Affinity Bioreagents polyclonal PPARγ 1,2 antibody was restricted to nuclei but differentiating preadipocytes (lipid accretion and C/EBPα positive) could not be distinguished from non-preadipocytes at either 1/50 or 1/250 dilution (not shown). Immunoreactivity for the the Santa Cruz PPARγ 1,2 antibody clearly distinguished preadipocytes so we used this antibody in the present study. Double staining simply involved lipid staining (no additional fixation) after peroxidase staining for either PPARγ, C/EBP's or the AD-3 antigen. On days 1, 3, and 6, cultures were reacted for the C/EBP isoforms, PPARγ, and the AD-3 antigen. Three dishes of each treatment were stained at each time point.

2.4. Western blot analysis of PPARγ proteins

At the designated times, the dishes were rinsed three times with 2 ml of ice-cold wash buffer (10 mmol/L Tris–HCl, pH 7.4, and 150 mmol/L NaCl) for one minute each followed by addition of 240 μl sample buffer 1 (50 mmol/L Tris, 200 mmol/L dithiothreitol (DTT), and 0.3% sodium dodecyl sulfate (SDS), pH 8.0) to one of the dishes. The cells were scraped and the cell lysate transferred into the next dish until all dishes were scraped into the buffer. The cell lysate was transferred into a microcentrifuge tube, heated for 5 min in boiling water, and chilled on ice for 5 min. Twenty-four microliters of sample buffer 2 (500 mmol/L Tris, 50 mmol/L $MgCl_2$, 1 mg/ml DNase 1, 0.25 mg/ml RNase A, pH 8.0) were added for 8 min and tubes kept on ice. Cellular proteins were precipitated by the addition of 1 ml of acetone and put on ice for 20 min. The tubes were centrifuged for 14,000*g* for 10 min at 4 °C, the supernatant discarded, and the pellet dried at room temperature for 5 min. The pellet was resuspended in 30–60 μl of sample buffer mix (40 mmol/L Tris, 7.92 mol/L urea, 0.06% SDS, 1.76% ampholytes, pH 3–10, 120 mmol/L DTT, and 3.2% Triton X-100). Five microliters were removed for measuring the protein concentration by Bio-Rad Protein Assay based on the Bradford method and the remainder frozen at −80 °C. At the day of immunoblot analysis, 200 μg of protein from each group were subjected to SDS–polyacrylamide gel electrophoresis in 7.5% gels. Cellular protein from one study was used to compare the polyclonal and monoclonal PPARγ 1,2 antibodies. After electrophoretic transfer to PVDF membranes, the Western blots were incubated overnight in Tris-buffered saline (20 mM Tris base and 8 g/L NaCl, pH 7.6) containing 0.1% Tween 20 and 5% milk (TBST-M). Blots were then incubated in either a monoclonal PPARγ 1,2 antibody (see above; Santa Cruz Biotechnology, Santa Cruz, California) at 1/300 dilution or rabbit polyclonal antibodies (Affinity BioReagents, Golden, CO) raised against either mouse PPARγ 1,2 (Catalogue # PA3-821) or mouse PPARγ2 (Catalogue # PA1-824) at 1/250 dilution. Subsequently blots were incubated with 1:5000 dilution of respective horseradish peroxidase polypeptidase conjugated second antibodies. The blots were washed extensively and immunoreactive proteins identified using an ECL kit and Hyperfilm ECL as described in manufacturer's protocol.

2.5. Statistics

Data were analyzed by a one-way analysis of variance (ANOVA) for main effects of cell culture treatment (SAS, 1985). The main effect of dam per se was not determined since cell culture treatments were not duplicated within a litter. As a result, each fetal S-V cell culture represented a separate dam for all measurements. Differences between means were determined by the least squares contrasts of the SAS (1985) procedure.

3. Results

3.1. Preadipocyte recruitment and C/EBPα immunocytochemistry

Early and late DEX treatment produced a significant but minimal increase in preadipocyte number (AD-3+ ; recruitment) in 50 day fetal S-V cell cultures (Fig. 1). The proportion of preadipocytes was only $1.2 \pm .3\%$ for early DEX treatment at day 6 (mean ± SEM of three

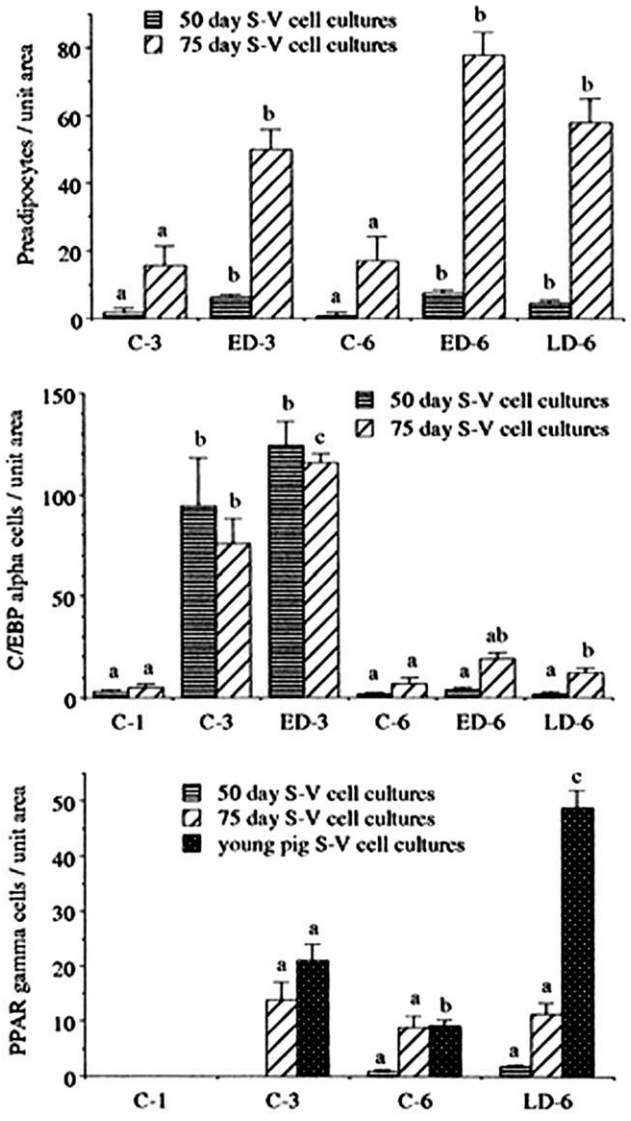

Fig. 1. Preadipocyte (AD-3+ cells) number (top panel) and number of C/EPPα (middle panel) and PPARγ (bottom panel) immunoreactive cells in 50 and 75 day fetal and young pig S-V cell cultures. Treatments included: ED-3, early DEX at day 3, i.e., FBS + DEX day 0–3; ED-6, early DEX at day 6, i.e., FBS + DEX day 0–3 followed by ITS day 3–6; LD-6, late DEX at day 6, i.e., FBS, day 0–3 followed by ITS + 10 nM DEX day 3–6; C-1, controls at day 1, i.e., FBS, day 0–1; C-3, controls at day 3, i.e., FBS, day 0–3; and C-6, controls at day 6, i.e., FBS day 0–3 followed by ITS day 3–6. Compared to control cultures, early DEX and late DEX increased the number of preadipocytes (top panel) in 50 and 75 day fetal S-V cultures. Early DEX increased the number of C/EBPα reactive cells (middle panel) in 75 but not 50 day fetal cultures. Late DEX increased the number of PPARγ reactive cells in young pig but not fetal S-V cell cultures (bottom panel). Note the large peak in C/EBPα reactive cell number during 50 and 75 day fetal cell cultures (middle panel). Values are least squares means ± SEM for 4–7 fetal S-V cell cultures or 4 young pig S-V cell cultures with 3–4 dishes in each replicate. Each S-V cell culture = S-V cells from 1 pig or 1 pool of fetal S-V cells/dam. [abc]Means in top and middle panels within staining groups and within day 6 and day 1–3 groups with different superscripts are significantly different ($P < .05$). [abc]Means in bottom panel within age of S-V cell donor groups with different superscripts are significantly different ($P < .05$).

cultures). In control (no DEX) 50 day fetal cell cultures there was an extremely low number of preadipocytes at day three and six (Fig. 1). Regardless of DEX treatment, preadipocytes were not stained for lipid at day three in 50 day fetal cell cultures but some preadipocytes were present as small clusters (data not shown). Early and late DEX increased absolute and relative (proportion) preadipocyte numbers in 75 day fetal S-V cultures (Figs. 1 and 2). Early DEX maximally increased absolute and relative numbers of preadipocytes by day 3 in 75 day cultures (Figs. 1 and 2). And, late DEX treatment resulted in a proportion of preadipocytes (Fig. 2) that was similar to the preadipocyte proportion induced by late DEX in young pig S-V cultures (Hausman, 2000). Most preadipocytes were lipid stained and present as large clusters in control 75 day fetal cultures at days 3 and 6 (data not shown) whereas there were no clusters of preadipocytes at day 1 of culture. Relative preadipocyte number was similar throughout the culture period in control 75 day fetal cultures (Fig. 2).

In 50 and 75 day fetal S-V cultures the absolute and relative number of C/EBPα reactive cells transiently increased between day 1 and day 3 of culture and then decreased markedly by day 6 (Figs. 1 and 2). Early DEX

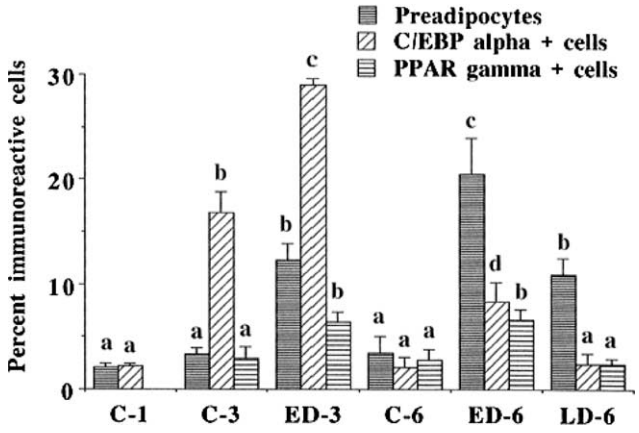

Fig. 2. Percentages or proportions of preadipocytes (AD-3+) and proportions of C/EBPα and PPARγ immunoreactive cells in 75 day fetal S-V cell cultures. Treatments included: ED-3, early DEX at day 3, i.e., FBS + DEX day 0–3; ED-6, early DEX at day 6, i.e., FBS + DEX day 0–3 followed by ITS day 3–6; LD-6, late DEX at day 6, i.e., FBS, day 0–3 followed by ITS + 10 nM DEX day 3–6; C-1, controls at day 1, i.e., FBS, day 0–1; C-3, controls at day 3, i.e., FBS, day 0–3; and C-6, controls at day 6, i.e., FBS day 0–3 followed by ITS day 3–6. Late DEX increased the proportion of preadipocytes but did not influence the proportion of PPARγ or C/EBPα reactive cells. Note that the marked decrease in the proportion of C/EBPα reactive cells (day three to day six) was not influenced by DEX treatment. Early DEX markedly increased the proportion of preadipocytes and to a lesser degree increased the proportion of PPARγ and C/EBPα reactive cells. Note that early DEX partially prevented the decrease in the proportions of C/EBPα reactive cells between day three and day six. Values are least squares means ± SEM for 4–7 fetal S-V cell cultures with 3–4 dishes in each replicate. [abcd]Means within staining groups with different superscripts are significantly different ($P < .05$).

treatment resulted in a higher number of C/EBPα reactive cells in 75 day cell cultures at days 3 and 6 compared to control cultures (no DEX; Figs. 1 and 2). The proportion of C/EBPα reactive cells after the third day of early DEX treatment was similar in 75 day fetal (Fig. 2) and young pig S-V cultures (Yu and Hausman, 1998; Hausman, 2000). Double staining clearly showed that all fat cells (lipid stained cells) and only fat cells were C/EBPα reactive in 50 and 75 day fetal cultures at day 6 (Fig. 3). Early and late DEX treatment had no influence on the number of C/EBPα reactive cells in 50 day cultures (Fig. 1).

In control and DEX treated 75 day fetal cultures, double staining showed that at 3 days of culture tight clusters of lipid stained cells were very reactive for C/EBPα whereas loosely clustered cells with either less or no lipid were less reactive for C/EBPα (Figs 4 and 5). There were no clusters of C/EBPα reactive cells at day 1 of culture. A considerable range in intensity of nuclear C/EBPα staining was also evident in 50 day fetal cultures at day 3 but there were no tightly clustered C/EBPα reactive cells and very few lipid stained C/EBPα reactive cells (data not shown).

Early and late DEX treatment of young pig cultures increased lipid accretion whereas late DEX maintained and early DEX increased C/EBPα reactive cell number (data not shown) as anticipated (Hausman, 2000; Yu and Hausman, 1998).

3.2. PPARγ immunocytochemistry

Immunocytochemistry showed that PPARγ reactivity was restricted to nuclei in permeabilized cells (Fig. 6)

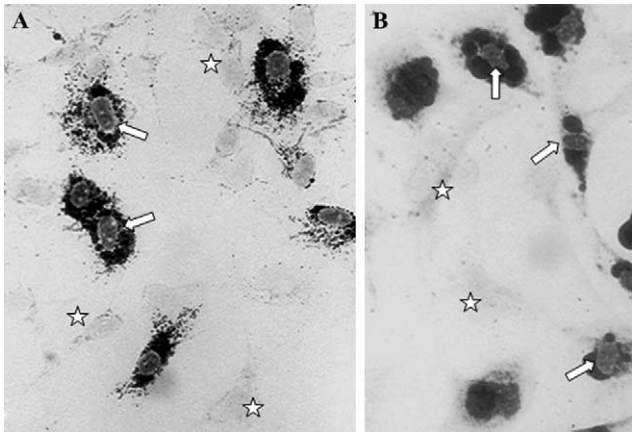

Fig. 3. Double-staining for lipid and either PPARγ (A) or C/EBPα (B) at day six in late DEX, i.e., ITS + DEX (A) and early DEX, i.e., FBS + DEX (B) treated 75 day fetal S-V cultures. Immunoreactivity was visualized by using a peroxidase staining kit. Lipid appears as the dark staining droplets in the cytosol. Note that all cells with lipid have PPARγ (A, white arrows) and C/EBPα reactive nuclei (B, white arrows). Also, note that reactive or stained nuclei have unreactive nucleoli (A). Stars (∗) indicate unreactive or unstained cells, A, 400×; B, 560×.

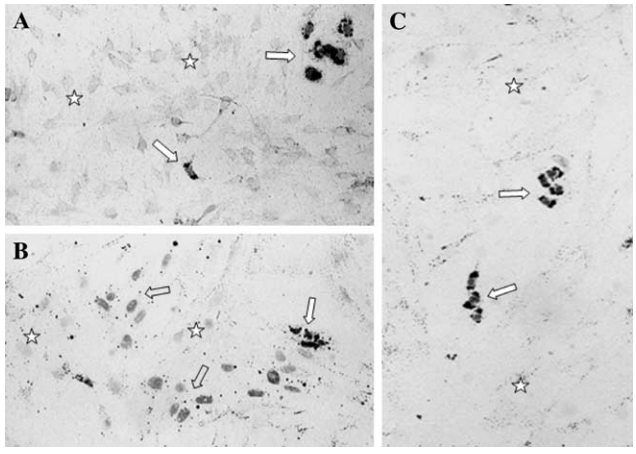

Fig. 4. Double-staining for lipid and either C/EBPα (A and B) or PPAR((C) in 75 day fetal S-V cultures at day three (A, C) and day six (B). Treatments included FBS, days 0–3 (A, C) and late DEX, i.e., ITS + DEX, days 3–6 (A). Immunoreactivity was visualized by using a peroxidase staining kit. Lipid appears as the dark staining droplets in the cytosol. All cells with lipid have C/EBPα and PPARγ reactive nuclei (white arrows). Note that C/EBPα reactive cells with little to no lipid (open arrows) are loosely clustered at day three (B) and decrease in number from day three (B) to day six (A). Only fat cells (white arrows) are PPARγ reactive at day 3 (C). In contrast to C/EBPα, there are no PPARγ reactive cells with little to no lipid (C). Stars (∗) indicate unreactive or unstained cells, A, 200×; B, 200×; C, 175×.

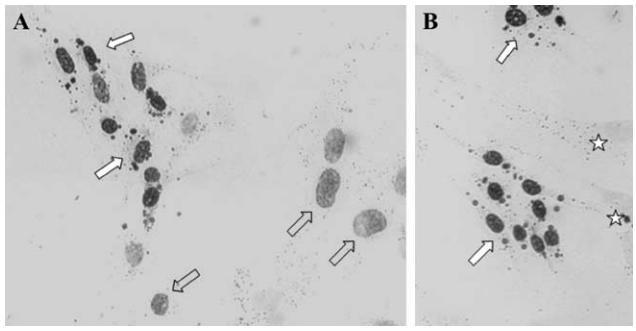

Fig. 5. Double-staining for lipid and C/EBPα in 75 day fetal S-V cultures at day three in FBS media. Immunoreactivity was visualized by using a peroxidase staining kit. Lipid appears as the dark staining droplets in the cytosol. Note that tightly clustered fat cells are intensely C/EBPα reactive (A and B, white arrows). Also, note the diverse morphologies of C/EBPα reactive nuclei in cells with little to no lipid (A, open arrows). Stars (∗) indicate unreactive or unstained cells, A, 300×; B, 300×.

whereas nuclear reactivity and minimal cytosolic reactivity was evident in cells that had not been permeabilized (data not shown). Regardless of DEX treatment, PPARγ reactive cells were not detectable at day one and three in 50 day fetal S-V cultures but were detected at day six (Fig. 1). PPARγ reactive cells were not present at day one in S-V cultures from 75 day fetuses and young pigs but were initially detected on the third day of culture (Fig. 1). Late DEX treatment increased PPARγ reactive cells in S-V cultures from young pigs but had no

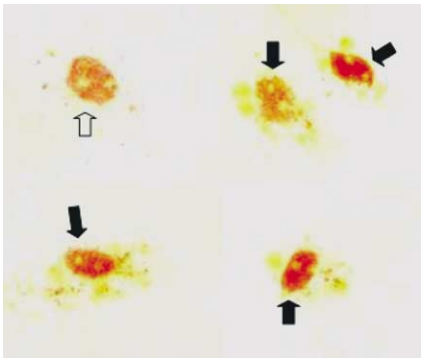

Fig. 6. PPARγ reactivity with no lipid staining or counter staining in 75 day fetal S-V cultures at day three in FBS media. Immunoreactivity was visualized by using a peroxidase staining kit. Note that PPARγ immunoreactivity is restricted to nuclei in fat cells (dark arrows) and in cells with little to no lipid (open arrow). Lipid has a light yellow color due to immunocytochemical processing. Nucleoli are devoid of PPARγ immunoreactivity, 400×.

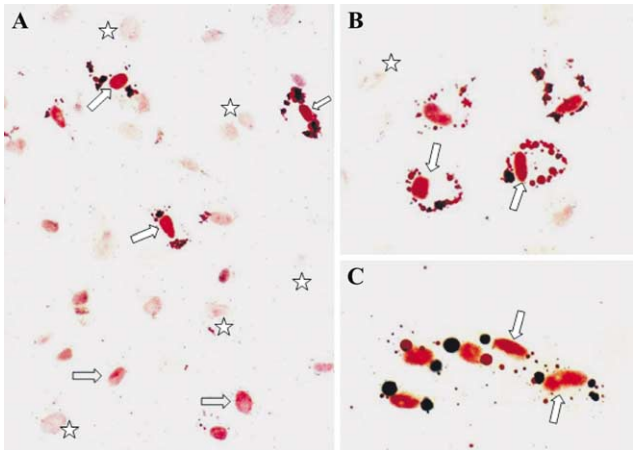

Fig. 7. Late DEX treated young pig S-V cultures at day 6 (A and B) and control 75 day fetal S-V cultures at day 3 (C) double-stained for lipid and PPARγ. Pig cultures were also counterstained with hematoxylin (A and B). Immunoreactivity was visualized by using a peroxidase staining kit. Lipid appears as the red to black staining droplets in the cytosol. Hematoxylin counterstaining results in lightly stained unreactive cells (∗). Note the PPARγ reactive differentiating cells (A and B, white arrows) and the PPARγ reactive cells with little to no lipid (A) indicative of DEX induced preadipocyte recruitment. Note the similar PPARγ reactivity within clusters of preadipocytes in DEX treated pig cultures (B, white arrows) and in control 75 day fetal cultures at day 3 (C, white arrows) associated with similar lipid accretion. Stars (∗) indicate unreactive cells, A, 400×; B, 550×; C, 600×.

influence on the relative and absolute number of PPARγ reactive cells in 50 and 75 day fetal cultures (Figs. 1 and 2). Early DEX increased the absolute and relative number of PPARγ reactive cells in 50 and 75 day fetal S-V cultures at day six (Fig. 2) but the number of PPARγ reactive cells was fivefold higher in 75 day fetal cultures since reactive cells/unit area for 50 and 75 day early DEX cultures were $2.9 \pm .5$ and 20 ± 1, respectively (means ± SEM of three experiments). PPARγ reactive cells were present as clusters of elongated or spherical cells in young pig and 75 day fetal cultures at day three regardless of DEX treatment (Figs. 4 and 7). And, double staining showed that all PPARγ reactive cells were lipid stained at 3 days of culture (Figs. 4 and 7). Furthermore, all fat cells were PPARγ reactive in late and early DEX treated young pig and fetal cultures at day 6 (Figs. 3 and 7). Fat cells in late DEX treated 75 day fetal cultures were similarly stained for PPARγ and there were no PPARγ reactive cells without lipid (Fig. 3). In contrast, there were PPARγ reactive cells with no lipid and various levels of fat cell PPARγ staining in late DEX treated young pig cultures (Fig. 7) which reflects preadipocyte recruitment and differentiation in these cultures.

The failure of late DEX treatment to increase PPARγ reactive cells and maintain C/EBPα reactive cells in fetal cultures clearly distinguishes fetal cultures from pig cultures (Figs. 2 and 4). However, the number of PPARγ reactive cells in 75 day fetal cultures at day 3 was predictive of the number of fat cells at day 6 (Figs. 1 and 2).

We reported that late DEX treatment had no influence on preadipocyte recruitment and C/EBPα cell number in S-V cell cultures derived from muscles of young pigs (Hausman et al., 2002). We report herein that after late DEX treatment the proportions of PPARγ reactive cells and fat cells in these "muscle" S-V cell cultures were $.74 \pm .1\%$ and $.1 \pm .1\%$, respectively (means ± SEM of two experiments). In contrast, the proportion of PPARγ reactive cells was higher in adipose tissue S-V cultures (young pig) after late DEX treatment, i.e., $9.3 \pm .4\%$ (means ± SEM of four experiments). Muscle and adipose tissue S-V cultures were directly compared in the experiments of muscle S-V cultures since the same pig provided tissue for both types of cultures. Total cell number was similar for muscle and adipose tissue S-V cell cultures after late DEX treatment.

3.3. C/EBPδ immunocytochemistry in fetal S-V cell cultures

All cells had C/EBPδ reactive nuclei regardless of fetal age, DEX treatment, and time in culture. Total cell counts and counts of C/EBPδ reactive cells were similar (data not shown).

3.4. Total cell numbers/unit area

Total cell numbers for 75 day S-V cultures at day 6 were similar for control (ITS) and late DEX treated cultures but were reduced ($P < 0.05$) by early DEX treatment, i.e., 368 ± 50 vs 565 ± 59 (means ± SEM of six cultures). And, as reported before (Hausman, 2000) late or early DEX treatment had no influence on total cell number in young pig S-V cultures (data not shown).

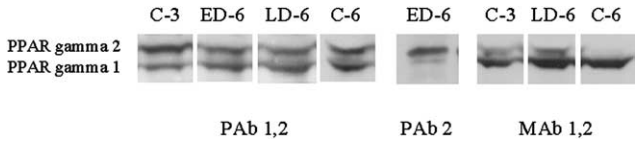

Fig. 8. Western blot analysis for PPARγ in whole-cell lysates of young pig S-V cell cultures. Similar levels of both PPARγ isoforms were detected with the rabbit polyclonal PPARγ 1,2 antibody (PAb 1,2) regardless of DEX treatment, i.e., I vs I + DEX and I vs F + DEX. Blots reacted with the monoclonal PPARγ 1,2 antibody (MAb 1,2) also showed that DEX treatment had little influence on PPARγ proteins. In blots reacted with the rabbit polyclonal PPARγ2 antibody (PAb 2) only the PPARγ2 isoform was detected. Treatments included : LD-6, ED-6, C-3, and C-6.

3.5. Western blot analysis of PPARγ proteins in young pig S-V cell cultures

Regardless of treatment, two PPARγ isoforms were detected with the rabbit polyclonal PPARγ 1,2 antibody (Affinity BioReagents; Fig. 8). The band corresponding to the PPARγ 2 isoform was not as obvious in blots reacted with the monoclonal PPARγ 1,2 antibody (Santa Cruz Biotechnology; Fig. 8). Regardless, late and early DEX treatment had little influence on the expression of total PPARγ proteins (Fig. 8). In blots reacted with the rabbit polyclonal PPARγ 2 antibody (Affinity BioReagents) only the PPARγ 2 isoform was detected (Fig. 8).

4. Discussion

In this study we demonstrated, for the first time, temporal relationships between C/EBPα and PPARγ protein expression and preadipocyte recruitment during porcine adipogenesis by examining S-V cell cultures that represented three levels of susceptibility to dexamethasone (DEX) induced preadipocyte recruitment and differentiation. Recruitment of preadipocytes by DEX clearly preceded lipid accretion and preadipocyte differentiation, i.e., expression of both C/EBPα and PPARγ proteins. In 50 day fetal S-V cultures DEX recruited a small number of preadipocytes but did not influence preadipocyte differentiation. Late DEX treatment in 75 day fetal S-V cultures recruited the same proportion of preadipocytes as in pig S-V cultures (Hausman, 2000) and early DEX treatment recruited a proportion of preadipocytes that was 50% of that in early DEX treated pig S-V cultures (Yu and Hausman, 1998). However, DEX had little to no influence on the expression of C/EBPα protein in fetal S-V cultures despite the influence on preadipocyte recruitment. For instance, there was little to no maintenance of C/EBPα protein expression after day three in early and late DEX treated fetal S-V cultures despite comparable expression of C/EBPα protein in fetal and pig S-V cultures by day three (Hausman, 2000; Yu and Hausman, 1998). In pig S-V cultures insulin maintains C/EBPα expression after day three with either early or late DEX treatment (Hausman, 2000; Yu and Hausman, 1998). Furthermore, high levels of the insulin receptor gene are evident in pig S-V cultures very early and throughout the culture period (McNeel et al., 2000). Possibly, C/EBPα protein expression is not maintained in fetal S-V cultures because fetal S-V cells do not express the insulin receptor gene and therefore are unable to respond to insulin. The development of insulin responsiveness elements like insulin receptors or signalling pathway components may lag behind C/EBPα protein expression in fetal S-V cultures. Regardless, these studies (present study; Hausman, 2000; Yu and Hausman, 1998) indicate that the maintenance of C/EBPα protein expression is critical to the ontogeny of glucocorticoid induced preadipocyte differentiation in the pig.

Most studies of PPARγ protein expression during preadipocyte differentiation in vitro have utilized Western blot analysis. In contrast, an immunocytochemical approach has been used in only three studies of PPARγ protein expression during preadipocyte differentiation (Caron et al., 2001; Csete et al., 2001; Tchoukalova et al., 2000). Although all three studies reported nuclear localization of PPARγ in differentiating preadipocytes, photomicrographs of immunoreactive preadipocytes were shown in only one study (3T3-F422A cell cultures; Caron et al., 2001). Nuclear localization of PPARγ protein in vitro indicates functional PPARγ protein since nuclear localization follows PPARγ activation by PPARγ ligands (Harris and Phipps, 2001; Jiang et al., 2000; Inoue et al., 2001). Considering the limited scope and nature of the previous immunocytochemical studies (Caron et al., 2001; Csete et al., 2001; Tchoukalova et al., 2000) the present study reports the first extensive and comprehensive immunocytochemical examination of PPARγ protein expression during preadipocyte differentiation. For instance, in the present study a unique experimental plan was coupled with immunocytochemistry for PPARγ, C/EBPα, another late preadipocyte protein, and two early appearing proteins (C/EBPδ and AD-3). Double staining for lipid and these proteins in fetal S-V cell cultures was used to indicate the relationship between the onset of preadipocyte lipid accretion and expression of these proteins. As a result, we demonstrate, for the first time, that reactivity for nuclear PPARγ protein was developmentally linked with lipid accretion in differentiating preadipocytes. The virtual absence of PPARγ reactive cells in 50 day fetal cultures at days one and three and at day one in 75 day fetal and young pig S-V cultures was associated with a near absence of fat cells in these cultures (present study; Hausman and Richardson, 1998). And, late DEX treatment increased the number of fat cells (Hausman

and Richardson, 1998) and the number of PPARγ reactive cells (present study) in young pig S-V cultures but has no influence on either in fetal S-V cultures (present study). Finally, the number of PPARγ reactive cells was greater in 75 day fetal cultures than in 50 day fetal cultures regardless of treatment or time in culture (present study) which reflects an increase in the number of adipogenic cells in adipose tissue between 50 and 75 days of fetal life (Hausman and Kauffman, 1986). Possibly, age dependent localization of PPAR?γ in S-V cells is involved in the onset of adipogenesis in fetal adipose tissue. However, there is little support for this hypotheses since there is no information on PPARγ? cellular localization in vivo. Regardless, glucocorticoids may induce similar numbers of preadipocytes in fetuses and young pigs but only preadipocytes induced in young pigs express PPARγ protein and maintain C/EBPα? protein expression which allows complete differentiation. Therefore, glucocorticoid regulation of preadipocyte adipogenesis in the pig may ultimately depend on PPARγ protein expression and nuclear localization.

The regulatory cascade that characterizes 3T3-L1 preadipocyte differentiation initially involves transient expression of C/EBPδ and β which activates expression of PPARγ (Wu et al., 1999; Yeh et al., 1995). Upon ligand activation, PPARγ subsequently induces C/EBPα expression concurrent with overt differentiation (Wu et al., 1999; Yeh et al., 1995). This pattern is not evident during pig adipogenesis since it is characterized by precocious C/EBPα expression (present study; Ding et al., 1999; Hausman, 2000; Lee et al., 1998) and continued expression of C/EBPβ and δ (present study; Ding et al., 1999; Hausman, 2000; Lee et al., 1998; McNeel et al., 2000; Yu and Hausman, 1998). Furthermore, we demonstrated, herein, that PPARγ protein expression does not precede C/EBPα expression during pig adipogenesis. And, PPARγ and C/EBPα gene expression in pig S-V cell cultures at day 0 (2% differentiated cells) was 35 and 40%, respectively, of the levels of gene expression at the end of culture (Ding et al., 1999). Thus, the temporal relationships between C/EBP isoform and PPARγ expression clearly distinguish pig preadipocyte differentiation from 3T3-L1 preadipocyte differentiation Furthermore, temporal aspects of preadipocyte differentiation in primary culture also distinguish rat and human preadipocyte differentiation from 3T3-L1 differentiation since C/EBPα, β, and γ proteins are expressed very early in rat S-V cultures (Lee et al., 1999) and the PPAR gene is expressed before confluency in human (Tchkonia et al., 2002) and rat S-V cultures (Hansen et al., 1998). The temporal patterns that diverge from the 3T3-L1 differentiation model may represent either species differences or differences between primary and clonal preadipocytes. However, expression of C/EBPδ protein precedes lipid accretion and expression of PPARγ and C/EBPα proteins in fetal (present study; Hausman and Yu, 1998) and pig S-V cell cultures (Hausman, 2000; Yu and Hausman, 1998) as in 3T3-L1 preadipocyte cultures (Yeh et al., 1995). Furthermore, the results of our study are consistent with the concept that cross-regulation between PPARγ and C/EBPα controls adipogenesis or maintains the differentiated state (Wu et al., 1999; Hamm et al., 1999) since fat cells expressed both proteins regardless of conditions (present study). Expression of PPARγ protein apparently rescued a small proportion of C/EBPα reactive cells in fetal S-V cultures and rescued all C/EBPα reactive cells in young pig S-V cultures. However, further studies are necessary to determine the molecular aspects of PPARγ and C/EBPα cross-regulation of pig preadipocyte differentiation.

Consideration of immunocytochemical studies of C/EBP isoforms in adipose tissue from 50 and 75 day fetuses is warranted since preadipocyte morphogenesis is similar in fetal S-V cultures (present study; Hausman, 1992) and fetal adipose tissue (Hausman and Kauffman, 1986). All cells in fetal adipose tissue sections (Lee et al., 1998) and fetal S-V cultures (present study) were uniformly reactive for C/EBPδ before and during adipogenesis. Differential C/EBPα staining and clusters of fat cells (strongly stained for C/EBPα) were evident in 75 day S-V cultures (present study) and 75 day fat tissue sections (Lee et al., 1998). However, there were no clusters of fat cells or C/EBPα reactive cells in 50 day fetal adipose sections (Lee et al., 1998) and 50 day S-V cultures (present study) despite differential C/EBPα staining. The onset of preadipocyte clustering in vitro may reflect the onset of preadipocyte clustering around developing blood vessels in vivo (Hausman and Kauffman, 1986). Clustered preadipocytes in vivo may retain morphological (Hausman and Richardson, 1998) and growth related biochemical traits in vitro (Chen et al., 1996). Consequently, clustered S-V cells or preadipocytes may be "activated" or conditioned by blood borne factors, like PPARγ activators, in vivo which could account for exclusive expression of PPARγ protein by clustered cells in vitro. However, the primary mechanisms or nature of conditioning have not been examined. Other factors such as cell to cell contact associated with cell clustering may also be involved in enhanced sensitivity to adipogenic factors or activators. Regardless, primary fetal S-V cultures are an exceptional in vitro system for the study of several fundamental aspects of adipogenesis in vivo.

Most Western blot studies of PPARγ expression during preadipocyte differentiation have used whole cell protein preparations (Adams et al., 1997; Hauser et al., 2000; Kim et al., 2000; Lee et al., 2001; Morrison and Farmer, 1999; Reginato et al., 1998; Stewart et al., 1999; Tchkonia et al., 2002; Tchoukalova et al., 2000). Nuclear localization of PPARγ cannot be assumed since cytosolic and nuclear localization of PPARγ protein has

been reported in culture studies of various cell types (Benson et al., 2000; Chattopadhyay et al., 2000; Nicholas et al., 2001) including 3T3-L1 preadipocytes (Thuillier et al., 1998). However, Western blot studies of PPARγ with whole cell and nuclear proteins from 3T3-L1 and -C2 preadipocytes have produced similar results (Bastie et al., 1999; Lee et al., 2001) Regardless, the present study and other studies of primary S-V cell cultures (Adams et al., 1997; Lee et al., 1998; Tchkonia et al., 2002; Tchoukalova et al., 2000) indicate that Western blotting consistently indicates more PPARγ protein than expected from measures of adipogenesis and PPARγ immunocytochemistry. In particular, Western blotting shows that PPARγ protein is detectable in preconfluent or undifferentiated cells in pig (Kim et al., 2001; present study) and human S-V cultures (Adams et al., 1997; Tchkonia et al., 2002) and PPARγ protein levels do not change appreciably with differentiation (present study; Lee et al., 1998; Tchkonia et al., 2002). In contrast, expression of PPARγ protein is not detectable in preconfluent or undifferentiated 3T3-L1 and 3T3-F442A cell cultures and PPARγ protein levels rapidly increase and plateau before adipogenesis is maximized (Caron et al., 2001; Hauser et al., 2000; Lee et al., 2001; Morrison and Farmer, 1999; Reginato et al., 1998; Stewart et al., 1999). PPARγ gene transcripts were detected in preconfluent or undifferentiated cells in rat (Hauser et al., 2001; Machinal-Quelin et al., 2002) and young pig (Ding et al., 1999) S-V cell cultures and high levels of PPARγ transcripts are evident early in culture which is consistent with PPARγ protein studies. Many or most cells in early S-V cell cultures may express the PPARγ gene but PPARγ protein may be localized in the cytosol and therefore not activated or functional. However, immunocytochemical and in situ hybridization studies of PPARγ in primary S-V cell cultures are necessary to examine this possibility.

The low level of PPARγ reactivity in 50 day fetal S-V cultures could be a function of an early age per se or indicate a very immature depot (low number of adipogenic cells). Since the intramuscular depot represents an immature depot we examined PPARγ reactivity in muscle or intramuscular S-V cell cultures from young pigs. The number of PPARγ reactive cells in intramuscular S-V cell cultures was extremely low and correlated with very low numbers of fat cells. Since the intramuscular S-V cell cultures were derived from young pigs, low levels of PPARγ reactivity probably indicate an immature depot regardless of age.

5. Summary/conclusions

This is the first immunocytochemical study of preadipocyte differentiation and expression of C/EBPα and PPARγ proteins in S-V cell cultures derived from adipose tissue before and during the onset of adipogenesis. These approaches revealed activated PPARγ protein and reduced basal numbers of preadipocytes and other lineage cells resulting in a more homogenous S-V cell population. Consequently, this study provides, for the first time, comprehensive and quantitative evidence that the onset of glucocorticoid regulated adipogenesis was associated with expression and nuclear localization of PPARγ protein. Furthermore, co-nuclear localization of C/EBPα and PPARγ proteins was associated with maintaining or regulating preadipocyte differentiation.

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